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# The seed of the Amazonian fruit *Couepia bracteosa* exhibits higher scavenging capacity against ROS and RNS than its shell and pulp extracts

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Among the large number of scientifically unstudied fruits from the Amazonia biome, *Couepia bracteosa* acts as an interesting source of bioactive compounds, such as phenolic compounds and carotenoids, which may be used for protecting human health against oxidative damage. For the first time, the phenolic compounds and carotenoids in extracts obtained from the pulp, shell and seeds of *C. bracteosa* fruits are reported, as well as their *in vitro* scavenging capacities against some reactive oxygen species (ROS) and reactive nitrogen species (RNS). The shell extract presented the highest phenolic compound and carotenoid contents (5540 and 328 μg per g extract, dry basis, respectively), followed by the pulp and seed extracts. The major phenolic compound was acacetin sulphate (one methoxy and two OH groups) (62%) in the shells; however, only seeds presented apigenin sulphate (three OH groups), in which it was the major compound (44%). The high content of apigenin sulphate may explain why the seed extract had the highest scavenging efficiency against all tested ROS/RNS among the studied extracts. Regarding carotenoids, all-*trans*-neochrome (17%) and all-*trans*-β-carotene (16%) were the major carotenoids in the pulp extracts, while all-*trans*-lutein (44%) was the most prevalent in the shell extracts and all-*trans*-α-carotene (32%) and all-*trans*-β-carotene (29%) were the major ones in the seed extracts.

## 1. Introduction

Brazilian Amazonia holds a great biodiversity of fruit species, comprising approximately 220 species of edible fruits that represent almost half of the diversity of native fruits in Brazil. These fruits are considered excellent sources of bioactive compounds that may be used for nutritional purposes and also for protecting human health against many diseases. Thus, enhancing the knowledge of the species, their chemical composition and their biological properties is one of the major challenges to improve their commercial value and rational exploitation.[1]

Recently, our research group has directed some efforts to study different fruits from Amazonia, concerning the characterization of bioactive compounds (phenolic compounds and carotenoids) and also the *in vitro* antioxidant potential against some physiologically relevant reactive oxygen and nitrogen species (ROS and RNS, respectively) to understand their nutraceutical potential and antioxidant benefits.[2–5] In biological systems, the production of ROS and RNS is important to maintain homeostasis. However, in the eventuality of an imbalance between the production of pro-oxidant reactive species and antioxidant defence capacity, as seen during the ageing process, the cellular components, such as lipids, proteins, DNA, and even tissue can be damaged (oxidative stress), resulting in several diseases, such as diabetes, cancer, allergies, inflammation, neurodegeneration and cardiovascular diseases.[6]

The *Couepia bracteosa* Benth species (Brazilian name: "pajurá") belongs to the Chrysobalanaceae R. Br. family. It is native to the Tropical Amazon and naturally found in the following Brazilian states: Amazonas, Amapá, Pará and Rondônia. The *C. bracteosa* tree is of medium size (up to 25 m high); the fruits are globose drupes of 8 to 12 cm long and 8 to 15 cm in diameter and weigh 80–200 g. The peel (exocarp) is dark-brown with a rough surface, covered with numerous white dots (lenticels). The pulp (mesocarp) is thick, fleshy and oily, yellow-brown in colour, with a grainy consistency, a sweet flavour vaguely reminiscent of nuts and a thick endocarp with a rough surface, dark-brown colour, abundant endosperm and

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just one large seed. The pulp is traditionally consumed *in natura*, as well as used to prepare different kinds of sweets, such as jams.[7]

To the best of our knowledge, no data related to the bioactive compounds or biological potential of *C. bracteosa* fruits have been published in the literature. However, tocopherols, flavonoids and derivatives and also triterpenes were reported for other species from the *Couepia* genus, such as *C. edulis*[8] and *C. paraensis.*[9–11] Furthermore, there are other reports in the literature that support the presence of interesting bioactive compounds in the *Couepia* genus. For example, the chemopreventive activity (induction of quinone reductase activity) of *C. ulei* compounds,[12] the antibacterial, antioxidant and cytotoxic activity against *Artemia salina* of *C. grandiflora* extracts,[13] and the anticancer activity (lyase inhibitors of DNA β-polymerase activity) of *C. polyandra.*[14]

In this paper, we are reporting, for the first time, the tentative identification and quantification (HPLC-DAD-MS$^n$) of the bioactive compounds (phenolic compounds and carotenoids) in seed, shell and pulp extracts obtained from *C. bracteosa* fruits, and also the antioxidant potential of each extract against some ROS and RNS with high relevance in biological systems: superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$). These results may help to stimulate the rational exploitation of natural resources from the Amazonian biome due to the small number of scientific studies about native fruits from that region, including the potential benefits not only for the local people, but also for the food, pharmaceutical and cosmetic industries.

## 2. Experimental

### 2.1. Chemicals

Nitroblue tetrazolium chloride (NBT), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), lucigenin, 30% hydrogen peroxide, sodium hypochlorite solution (4% available chlorine), dihydrorhodamine 123 (DHR), 4,5-diaminofluorescein (DAF-2), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), quercetin, acacetin, apigenin, all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin, all-*trans*-β-carotene, dimethyl sulfoxide (DMSO), ethanol, methanol, methyl *tert*-butyl ether (MTBE), acetonitrile and all other chemical salts and solvents of analytical grade were obtained from Sigma-Aldrich (St. Louis, USA). Ultrapure water was obtained from the arium® pro system (Sartorius, Germany). All phenolic compounds and carotenoids standards showed at least 95% purity, as determined by HPLC-DAD.

### 2.2. *C. bracteosa* samples and extract preparation

The *C. bracteosa* fruits were acquired in three street markets (~1 kg) in Manaus, Amazonas, Brazil (03°06′07″S and 60°01′30″W). All the fresh and ripe fruits (~3 kg) were combined, washed with distilled water and the pulp, shell and seeds were manually separated to prepare the three different extracts. Approximately 50 g of pulp or shell or seeds was submitted to extraction with absolute ethanol in a mass/solvent ratio of 1 : 10 (w/v), for 4 h at room temperature (25 °C), protected from light and under agitation (~80 rpm) using a magnetic stirrer. The extracts were vacuum-filtered (Whatman filter paper n° 4) and the solvent was evaporated under reduced pressure ($T$ < 40 °C).[4] All concentrated extracts were freeze-dried, transferred to amber glass bottles and stored at −20 °C for further analysis.

### 2.3. HPLC-DAD-MS$^n$ analysis of phenolic compounds and carotenoids

**2.3.1. Equipments.** The identification and quantification of phenolic compounds in all extracts was performed in an Accela HPLC system (Thermo Fisher Scientific, San Jose, CA) equipped with a quaternary pump (Accela 600), a DAD detector and an auto-sampler cooled to 5 °C. The equipment was also connected in series to a LTQ Obritrap™ XL mass spectrometer (MS) (Thermo Fisher Scientific, San Jose, CA) with an electrospray ionization source (ESI), and a hybrid system combining a linear ion-trap and the Orbitrap as the *m*/*z* analyzer. The identification of carotenoids was performed in a Shimadzu HPLC (Kyoto, Japan) equipped with a quaternary pump (LC-20AD), a degasser unit (DGU-20A5), a Rheodyne injection valve with a 20 μL loop, a DAD detector (SPD-M20A), and connected in series to a MS from Bruker Daltonics (AmaZon speed ETD, Bremen, Germany) with atmospheric pressure chemical ionization (APCI) and an ion-trap as the *m*/*z* analyzer. The quantification of carotenoids was carried out in a LaChrom HPLC system (D-700, Merck Hitachi Ltd, Tokyo, Japan) equipped with a quaternary pump (L-7100) and a DAD detector (L-7455). For all the chromatographic analysis, samples and solvents were filtered using membranes of 0.22 and 0.45 μm, respectively, both from Millipore (Billerica, MA, USA).

**2.3.2. Determination of phenolic compounds and carotenoids from *C. bracteosa* extracts.** The phenolic compounds were analysed after dissolving 50 mg of the freeze-dried extract from each fruit part in methanol/water (80 : 20, v/v) and the compounds were separated on a $C_{18}$ Synergi Hydro column (4 μm, 250 × 4.6 mm, Phenomenex), at 0.9 mL min$^{-1}$, at a column temperature of 29 °C and with a mobile phase consisting of water/formic acid (99.5 : 0.5, v/v) and acetonitrile/formic acid (99.5 : 0.5, v/v) in a linear gradient.[15] The column eluate was split to allow only 0.3 mL min$^{-1}$ to enter the ESI interface. The UV-Vis spectra were obtained between 200 and 600 nm, and the chromatograms were processed at 280, 320 and 360 nm. Mass spectra were obtained after ionization in an ESI source in the negative ion mode, with a scan range from *m*/*z* 100 to 1000, and the MS parameters were set at the same conditions as described in our previous work.[4] Phenolic compounds were tentatively identified based on the following data: elution order, retention time of the peaks and characteristics of the UV-visible and mass spectra in comparison with authentic standards (data not shown) analysed under the same conditions and data available in the literature.[3–5,15,16] The quantification was carried out by comparison to external

 

analytical curves (1 to 100 μg $mL^{-1}$, in duplicate) using five points for the standards apigenin (at 339 nm, $r^2 \geq 0.99$) and acacetin (at 327 nm, $r^2 \geq 0.99$).

For the carotenoid analysis, 50 mg of each freeze-dried extract of *C. bracteosa* was dissolved in acetone and subjected to exhaustive extraction, liquid-liquid partition, saponification and drying under $N_2$ flow, following the same procedures described in detail by Ribeiro *et al.*[4] The dried saponified carotenoid extracts were re-suspended in methanol/MTBE (70 : 30, v/v) and injected into the chromatographic systems. The carotenoids were separated on a $C_{30}$ YMC column (5 μm, 250 mm × 4.6 mm) with a linear gradient of methanol and MTBE at 0.9 mL $min^{-1}$ and with a column temperature set at 29 °C.[15] The UV-Vis spectra were recorded between 200 and 600 nm and the chromatograms were processed at 450 nm. The column eluate was directed to the APCI interface and the mass spectra were obtained after ionization in the positive ion mode, with a scan range from *m*/*z* 100 to 800 and MS parameters set as described by Chisté and Mercadante.[15] The carotenoids were tentatively identified according to the following combined information: elution order, retention time, co-chromatography with authentic standards, UV-visible spectra ($\lambda_{max}$, spectral fine structure (%III/II), and peak *cis* intensity (% $A_B/A_{II}$)) compared with data available in the literature.[4,15,17] The characterization of each *cis*-isomer of the carotenoids was based on the observed decrease in the %III/II values and increase in the $\%A_B/A_{II}$ values (≈7–11% = 9-*cis*; ≈45% = 13-*cis* *e* ≈ 56% = 15-*cis* carotenoid) as the *cis* double bond moves from the end to the centre of the molecule.[17] The carotenoids were quantified by HPLC-DAD by comparison to standards using five-point external analytical curves (0.5–30 μg $mL^{-1}$, in duplicate) for all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene. All other carotenoids (including epoxy and *cis* isomers) were estimated using the curve of the corresponding all-*trans*-carotenoid.

The contents of phenolic compounds and carotenoids of all extracts, determined by HPLC-DAD, were expressed as μg $g^{-1}$ of extract (dry basis), considering three independent extraction procedures ($n = 3$).

### 2.4. ROS- and RNS-scavenging assays

The scavenging assays against all ROS and RNS were carried out in a microplate reader (Synergy HT, Biotek, Vermont, USA) equipped with a thermostat and detection systems for the measurement of fluorescence, UV-Vis and chemiluminescence. Quercetin was used as a positive control in all assays and its $IC_{50}$ values were similar to those already reported by our research group.[2–4] DMSO was used to solubilise all extracts of *C. bracteosa* fruit in all assays, except for the HOCl-scavenging assay (ethanol was used). Additional experiments were performed with all extracts to ensure the results were not affected by any interference of solvents or fluorescence/chemiluminescence/absorbance response of the extracts (data not shown). The $IC_{50}$ values (*in vitro* inhibitory concentration of the extract which is able to reduce, by 50%, the effect of ROS or RNS) were calculated from the curves of percentage of inhibition *versus* antioxidant concentration using GraphPad Prism 6 software. For each assay, four independent experiments were performed, using six different concentrations.

**2.4.1. $O_2^{\bullet-}$-scavenging assay.** The non-enzymatic system NADH/PMS/$O_2$ was used to generate $O_2^{\bullet-}$, which promotes the reduction of NBT into a purple coloured diformazan compound. This reaction was followed by spectrophotometry, at 560 nm, for 2 minutes, by monitoring the effect of each *C. bracteosa* extract and the positive control against the $O_2^{\bullet-}$-induced reduction of NBT.[3] The scavenging capacities were expressed as the percentage of inhibition of the NBT reduction to diformazan.

**2.4.2. $H_2O_2$-scavenging assay.** The effect of each *C. bracteosa* extract and the positive control against the $H_2O_2$-induced oxidation of lucigenin was monitored by chemiluminescence at 37 °C and the signal was detected immediately after the introduction of the plate in the reader.[3] The scavenging capacities were expressed as the percentage of inhibition of $H_2O_2$-induced oxidation of lucigenin.

**2.4.3. HOCl-scavenging assay.** HOCl was immediately prepared before the assay using a NaOCl solution 1% (w/v) and adjusting to pH 6.2 with a diluted solution of $H_2SO_4$. The concentration of HOCl obtained after the reaction was determined by spectrophotometry at 235 nm using a molar absorption coefficient of 100 $M^{-1}$ $cm^{-1}$. The capacity of each extract and the positive control to scavenge HOCl was determined by monitoring the HOCl-induced oxidation of DHR (non-fluorescent) to rhodamine 123 (fluorescent).[3] The scavenging capacities were expressed as the percentage of inhibition of HOCl-induced oxidation of DHR.

**2.4.4. $^{\bullet}$NO-scavenging assay.** The antioxidant effect of the *C. bracteosa* extracts and the positive control was measured by monitoring the oxidation of DAF-2 to the fluorescent triazolofluorescein (DAF-2 T) induced by $^{\bullet}$NO, which was generated by the decomposition of NOC-5. The fluorescence signal was followed during 30 minutes of incubation at 37 °C.[3] The scavenging capacities were expressed as the percentage of inhibition of $^{\bullet}$NO-induced oxidation of DAF-2.

**2.4.5. $ONOO^-$-scavenging assay.** $ONOO^-$ was synthesized as previously described by Fernandes, Gomes, Costa & Lima.[18] The capacity of each extract and the positive control in scavenging $ONOO^-$ was determined by monitoring the $ONOO^-$-induced oxidation of non-fluorescent DHR to the fluorescent rhodamine 123.[3] Parallel experiments simulating physiological concentrations of $CO_2$ were performed using 25 mM $NaHCO_3$. The scavenging capacities were expressed as the percentage of inhibition of $ONOO^-$- induced oxidation of DHR.

## 3. Results and discussion

### 3.1. Phenolic compounds and carotenoids from *C. bracteosa* extracts

The phenolic compounds (Fig. 1) and carotenoids (Fig. 2) of all *C. bracteosa* extracts in this study were separated, identified and quantified by HPLC-DAD-$MS^n$. In relation to the phenolic

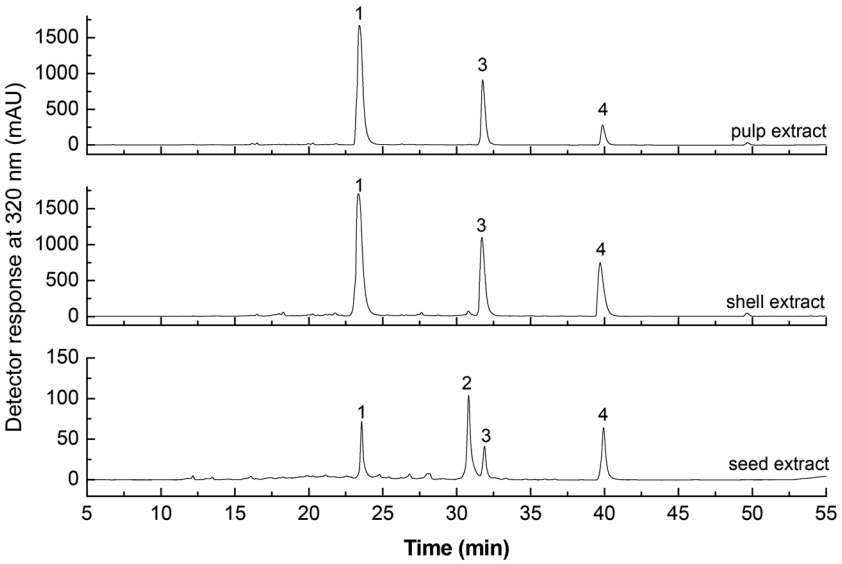


**Fig. 1** HPLC-DAD chromatogram of the phenolic compounds in pulp, shell and seed extracts of *Couepia bracteosa* fruits. Peak characterization is given in Table 1.

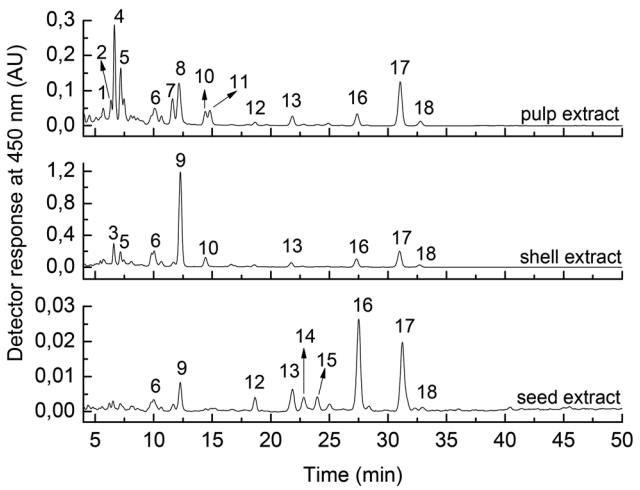


**Fig. 2** HPLC-DAD chromatogram of the carotenoids in pulp, shell and seed extracts of *Couepia bracteosa* fruits. Peak characterization is given in Table 2.

compound identification, as seen in Table 1, peak 1 and 4 were assigned as acacetin sulphate, probably positional isomers, since both peaks showed $[M - H]^-$ at *m*/*z* 363, exhibited a neutral loss of 80u (*m*/*z* 283) in the $MS^2$ spectra, which indicates the loss of a sulphate moiety esterified to acacetin $[M - H - SO_3]^-$, and had fragments at *m*/*z* 268 $[M - H - SO_3 - CH_3]^-$, indicating the loss of a methyl group after losing the sulphate moiety. The identity was confirmed by the same MS characteristics observed after analyzing the acacetin standard (data not shown). Peak 2 showed $[M - H]^-$ at *m*/*z* 349 with a high intense loss of a sulphate moiety $[M - H - SO_3]^-$ (*m*/*z* 269) and was tentatively identified as apigenin sulphate, since the fragmentation of *m*/*z* 269 ($MS^3$) presented the same fragmentation pattern of the authentic standard of apigenin [neutral losses of 28u (CO) and 44u ($CO_2$)] (data not shown). Peak 3 presented $[M - H]^-$ at *m*/*z* 377 and was tentatively identified as the oleuropein derivative after comparing the $MS^2$ and $MS^3$ features with those data already well described in the literature.[16,19]

Sulphate esters of flavonoids are relatively rare compounds and their functional significance in plant tissues is not clear. They are found mainly in species occurring in coastal and swampy areas rich in mineral salts, as well as in plants occurring in arid habitats.[20,21] In such plants, the binding reaction of inorganic sulphates to flavonoids is probably one of the mechanisms connected with the biochemical adaptation of species to their environments.[21] Although the phenolic profile of *C. bracteosa* fruits was reported for the first time in this study, there is another report available, in which two active compounds were identified in the ethyl acetate extracts of *C. ulei* stems: *erythro*-2,3-bis(4-hydroxy-3-methoxyphenyl)-3-ethoxypropan-1-ol and a known compound, evofolin-B, along with five inactive compounds (betulinic acid, oleanolic acid, pomolic acid, (+)-syringaresinol and ursolic acid).[12]

Regarding the carotenoid profile, 18 compounds were separated and tentatively identified and quantified (Table 2). The $MS^2$ experiments confirmed the assignment of the protonated molecule ($[M + H]^+$) of all identified peaks through the fragments expected for the carotenoid polyene chain and functional groups, along with the UV-Vis spectra features.[15,17] The carotenoid composition was slightly different for each extract, with a predominance of xanthophylls with one to three hydroxyl groups (OH), mostly with one or two epoxide groups. The identification of all-*trans*-lutein (peak 9), all-*trans*-zeaxanthin (peak 10), all-*trans*-β-cryptoxanthin (peak 13) and all-

 

**Table 1** Phenolic compounds in pulp, shell and seed extracts obtained from *Couepia bracteosa* fruits, as tentatively identified by their chromatographic, UV-Vis and mass spectroscopy characteristics (HPLC-DAD-ESI-MS$^n$)

| Peaks | $t_R$ range[a] (min) | $\lambda_{max}$[b] (nm) | $[M-H]^-$ ($m/z$) | Fragments[c] ($m/z$) | Compounds | Concentration[d] (μg per g extract) Pulp | Shell | Seed |
|---|---|---|---|---|---|---|---|---|
| 1 | 23.4–23.6 | 270, 330, 338 | 363.0174 | $MS^2$ [363]: 348, 320, **283**, 268<br>$MS^3$ [363 → 283]: **268**, 255, 239, 165 | Acacetin sulphate[e] | 4461 ± 195 | 7958 ± 287 | 2289 ± 52 |
| 2 | 30.6–30.8 | 268, 320(sh), 344 | 349.0042 | $MS^2$ [349]: 331, 283, **269**, 239, 211<br>$MS^3$ [349 → 269]: 241, **225**, 197, 149 | Apigenin sulphate[f] | nd | nd | 5058 ± 107 |
| 3 | 31.7–31.9 | 270, 330 | 377.0333 | $MS^2$ [377]: **362**, 334, 297, 282, 252<br>$MS^3$ [377 → 362]: 333, 298, 281, **252** | Oleuropein aglycon[f] | 2037 ± 45 | 4749 ± 159 | 1393 ± 74 |
| 4 | 39.7–39.9 | 270, 330, 342 | 363.0187 | $MS^2$ [363]: **348**, 283, 268, 253, 225<br>$MS^3$ [363 → 348]: 330, 320, **268**, 238 | Acacetin sulphate[e] | 654 ± 16 | 3914 ± 37 | 2702 ± 81 |
| | | | | | **Sum of phenolic compounds** | **7152 ± 1385** | **16 621 ± 1612** | **11 443 ± 1099** |

[a] Retention time on the $C_{18}$ Synergi Hydro (4 μm) column. [b] Solvent: gradient of 0.5% formic acid in water and acetonitrile with 0.5% formic acid. [c] In the $MS^2$ and $MS^3$, the most abundant ions are shown in boldface. [d] Mean ± standard deviation ($n$ = 3, dry basis). [e] The peaks were quantified as equivalent to acacetin. [f] The peaks were quantified as equivalent to apigenin. nd = not detected.

**Table 2** Chromatographic, UV-Vis and mass spectroscopy characteristics (HPLC-DAD-APCI-MS$^n$) and contents of carotenoids in pulp, peel and seed extracts obtained from *Couepia bracteosa* fruit

| Peak | Carotenoid | HPLC-DAD-APCI-MS$^n$ $t_R$[a] (min) | $\lambda_{max}$[b] (nm) | %III/II | $\%A_B/A_{II}$ | $[M+H]^+$ ($m/z$) | $MS^2$ ($m/z$) | Concentration[c] (μg per g extract) Pulp | Shell | Seed |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *cis*-Neochrome[1] | 5.6–5.9 | 300, 390, 417, 441 | 50 | 39 | nd | nd | 8.3 ± 0.7 | nd | nd |
| 2 | *cis*-Neochrome [1] | 6.2–6.5 | 300, 390, 417, 442 | 75 | 23 | 601 | 583, 565, 547, 491, 221 | 7 ± 1 | nd | nd |
| 3 | Not identified[1] | 6.4–6.6 | 420, 448 | nc | 0 | nd | nd | nd | 30 ± 1 | nd |
| 4 | All-*trans*-neochrome[1] | 6.6–6.9 | 399, 421, 448 | 94 | 0 | 601 | 583, 565, 547, 491, 221 | 22 ± 3 | nd | nd |
| 5 | 9-*cis*-Neochrome[1] | 7.1–7.3 | 304, 398, 421, 448 | 89 | 7 | 601 | 583, 565, 547, 491, 221 | 14 ± 2 | 20 ± 1 | nd |
| 6 | All-*trans*-luteoxanthin[1] | 10.1–10.3 | 399, 421, 447 | 100 | 0 | 601 | 583, 565, 491, 221 | 9.5 ± 0.6 | 24 ± 2 | 1.7 ± 0.5 |
| 7 | Not identified[1] | 11.6–11.8 | 400, 427, 451 | 70 | 0 | 585 | 567, 549, 493, 221 | 7.4 ± 0.6 | nd | nd |
| 8 | Not identified[1] | 12.1–12.3 | 313, 400, 427, 452 | 60 | 7 | 585 | 567, 549, 493, 221 | 18 ± 1 | nd | nd |
| 9 | All-*trans*-lutein[2] | 12.0–12.3 | 420, 444, 472 | 50 | 0 | 569 | 551, 533, 477 | nd | 146 ± 11 | 2.5 ± 0.6 |
| 10 | All-*trans*-zeaxanthin[3] | 14.2–14.4 | 420, 450, 476 | 12 | 10 | 569 | 551, 533, 477 | 3.3 ± 0.2 | 20 ± 2 | nd |
| 11 | Not identified[1] | 14.9–15.1 | 324, 410, 440, 468 | 54 | 11 | 585 | 567, 549, 493, 475, 221 | 6.2 ± 0.2 | nd | nd |
| 12 | Not identified[1] | 18.5–18.7 | 321, 420, 445, 472 | 50 | 23 | 553 | 535, 517, 497, 461 | 1.34 ± 0.03 | nd | 1.60 ± 0.08 |
| 13 | All-*trans*-β-cryptoxanthin[4] | 21.8–22.0 | 420, 450, 475 | 0 | 0 | 553 | 535, 473, 461 | 3.62 ± 0.03 | 7.4 ± 0.4 | 2.3 ± 0.3 |
| 14 | Not identified[1] | 22.6–23.0 | 400, 425, 450 | nc | 0 | 553 | 535, 473, 461 | nd | nd | 2.3 ± 0.3 |
| 15 | *cis*-α-Carotene[1] | 23.7–24.0 | 330, 418, 438, 468 | 54 | 16 | 537 | 481, 444, 413 | nd | nd | 2.4 ± 0.6 |
| 16 | All-*trans*-α-carotene[1] | 27.4–27.7 | 420, 445, 473 | 50 | 0 | 537 | 481, 444, 413 | 5.7 ± 0.2 | 23 ± 3 | 11 ± 2 |
| 17 | All-*trans*-β-carotene[1] | 31.4–31.9 | 420, 450, 477 | 28 | 0 | 537 | 457, 444, 413 | 20.7 ± 0.6 | 52 ± 4 | 10 ± 3 |
| 18 | 9-*cis*-β-Carotene[1] | 33.0–33.7 | 328, 420, 446, 470 | nc | 11 | 537 | 457, 444, 413 | 2.64 ± 0.07 | 6 ± 1 | 1.1 ± 0.2 |
| | | | | | | | **Total carotenoids (μg g$^{-1}$)** | **130 ± 7** | **328 ± 41** | **34 ± 4** |

[a] Retention time on the $C_{30}$ column. [b] Linear gradient of methanol/MTBE. [c] Mean ± standard deviation ($n$ = 3, dry basis). nc = not calculated. nd = not detected. The peaks were quantified as equivalent to all-*trans*-β-carotene[1], all-*trans*-lutein[2], all-*trans*-zeaxanthin[3] and all-*trans*-β-cryptoxanthin[4].

*trans*-β-carotene (peak 17) was positively confirmed through co-elution with authentic standards, as well as by comparison of their UV-vis and MS spectra features with standards. Peaks 2, 4 and 5 (Table 2) presented the same MS and $MS^2$ spectra characteristics: $[M + H]^+$ at *m*/*z* 601, and three consecutive neutral losses of water from the protonated molecule were observed at *m*/*z* 583 $[M + H\text{-}18]^+$, *m*/*z* 565 $[M + H\text{-}18\text{-}18]^+$ and *m*/*z* 547 $[M + H\text{-}18\text{-}18\text{-}18]^+$, as well as a fragment at *m*/*z* 491 $[M + H\text{-}18\text{-}92]^+$ resulting from an additional loss of the toluene moiety (92u) from the polyene chain. Peak 6 also presented $[M + H]^+$ at *m*/*z* 601, but only two consecutive losses of water were observed in the $MS^2$ spectrum (*m*/*z* 583 and *m*/*z* 565). Peaks 7, 8, and 11 showed $[M + H]^+$ at *m*/*z* 585 and the presence of two OH attached to the carotenoid molecules was demonstrated by the consecutive losses of two water moieties in its $MS^2$ spectra (*m*/*z* 567 and *m*/*z* 549). In addition, the fragment at *m*/*z* 221 was observed in all the peaks that correspond to an epoxy substituent in a β-ring with a OH group.[17] Moreover, all these previous peaks showed a hypsochromic shift of 10 nm (peak 11) and 25–30 nm (peaks 7 and 8) in relation to β-carotene (450 nm, peak 17), which indicates the presence of a 5,6-epoxy or 5,8-furanoid group in the carotenoid structures. Although 5,8-epoxides were already found in other Amazonian fruits, such as buriti, marimari, palm oil, peach palm, physalis and tucuma,[17] it is not possible to ensure that a 5,6-epoxy to 5,8-furanoid rearrangement did not occur during the preparation and storage of the extracts obtained from *C. bracteosa*. However, peaks 7, 8 and 11 were assigned as "not identified" due to the lack of visible fragments at *m*/*z* 205 (β-ring with an epoxy group) in their $MS^2$ spectra. In the same sense, other minor peaks (peaks 3, 12 and 14) were also assigned as "not identified" due to the lack of consistent data between their UV-visible and MS spectra features as compared with data available in the literature to ensure their tentative identification.

Peaks 15 to 18 belong to the carotene group since they all presented $[M + H]^+$ at *m*/*z* 537 with a characteristic neutral loss of toluene at *m*/*z* 444 $[M + H\text{-}92]^+$. The presence of fragments at *m*/*z* 481 and *m*/*z* 444 (peaks 15 and 16) corresponds to the respective losses of ε-ring and toluene as in α-carotene and its isomers.[22] Additionally, the assignment of all-*cis*-isomers took into account that the spectral fine structure (%III/II) decreases and the intensity of the *cis*-peak ($\%A_B/A_{II}$) increases as the *cis*-double bond is getting closer to the centre of the molecule.

The shell extract of *C. bracteosa* fruit presented the highest phenolic compound and carotenoid content (16 621 and 328 μg per g extract, respectively) (Tables 1 and 2), followed by the pulp and seed extracts. The major phenolic compound identified in the pulp and shell extracts was acacetin sulphate, accounting for 62 and 48% of the total sum of the identified phenolic compounds, respectively, followed by the oleuropein derivative compound (28% in both cases), while apigenin sulphate was only found in the seed extracts and it was the major compound (5058 μg per g extract), accounting for 44% of the total sum of the phenolic compounds. Regarding carotenoids, all-*trans*-neochrome and all-*trans*-β-carotene were the major compounds identified in the pulp extracts (22 and 21 μg per g extract, respectively), while all-*trans*-lutein (146 μg per g extract) was the major compound in the shell and all-*trans*-α-carotene and all-*trans*-β-carotene were the major ones in the seed extracts (11 and 10 μg per g extract, respectively).

### 3.2. Scavenging capacities of *C. bracteosa* extracts against ROS and RNS

According to Table 3, all *C. bracteosa* extracts were able to scavenge the tested ROS and RNS in a concentration-dependent manner (Fig. 3). However, their scavenging capacities did not seem to be directly related to the total yield of phenolic compounds or carotenoids found in each extract (Tables 1 and 2).

The seed extract was the most efficient against all tested ROS and RNS notwithstanding its lower contents of phenolic compounds (11 443 μg $g^{-1}$) and carotenoids (34 μg $g^{-1}$) compared to the amounts found in the shell extract (Tables 1 and 2). The high scavenging capacity of the seed extract of *C. bracteosa* fruits may probably be attributed to the presence of apigenin sulphate, even at low concentration (Fig. 1, Table 1), since this compound was only detected in the extracts obtained from the seeds. Some studies have already reported the high antioxidant properties of apigenin.[23–25] Additionally, the other identified phenolic compounds (acacetin and oleuropein) are promising bioactive compounds and they have also been studied due to their beneficial effects to human health.[26–28]

The seed extracts showed a high scavenging capacity against $O_2^{\bullet-}$ (Fig. 3a) with an $IC_{50}$ of 11.5 μg $mL^{-1}$, while the pulp and shell extracts showed no activity against this ROS, at the highest tested concentration (1000 μg $mL^{-1}$). Although $O_2^{\bullet-}$ is not considered as a potent pro-oxidant species *per se*, it represents a key point in the oxidative stress as a primary generated ROS. $O_2^{\bullet-}$ production plays an important role in cellular signalling and in the development of pathophysiological conditions, such as hypertension, ischemia-reperfusion, inflammation, and atherosclerosis.[6] In our study, the scavenging capacity of the *C. bracteosa* seed extract against $O_2^{\bullet-}$ was higher than that found for quercetin (positive control) ($IC_{50}$ = 14.2 μg $mL^{-1}$) and also higher than those reported for water and ethanol/water extracts of *Caryocar vilosum* fruit pulp,[3] another Amazonian fruit, pulp and peel extracts of *Psidium cattleianum* fruits,[4] and infusion and decoction extracts obtained from artichoke leaves.[29]

Once formed, $O_2^{\bullet-}$ can be physiologically dismutated to $H_2O_2$ by the action of the superoxide dismutase (SOD) enzyme, or spontaneously under acid conditions. $H_2O_2$, although not a free radical, presents a high reactive potential, since it has a long lifetime. It is able to cross cell membranes and therefore may be potentially cytotoxic, mainly due to its participation in $^{\bullet}OH$ generation by the reactions catalysed by iron and/or copper ions (Fenton and Haber–Weiss reactions).[30] Again, the seed extract of *C. bracteosa* was the most efficient extract against $H_2O_2$ (Fig. 3b), with an $IC_{50}$ at high μg $mL^{-1}$ level (426 μg $mL^{-1}$), followed by the shell extract (894 μg $mL^{-1}$), while the pulp extract could decrease the oxidizing effect of $H_2O_2$ only by 29%, at the highest tested concentration (1000 μg

 

Table 3 Scavenging capacities of the pulp, shell and seed extracts obtained from *Couepia bracteosa* fruits against superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$)

| | $IC_{50}$ (μg $mL^{-1}$) ($n = 4$)[a] | | | |
|---|---|---|---|---|
| | *Couepia bracteosa* extracts | | | Positive control |
| Reactive species | Pulp | Shell | Seed | Quercetin |
| ROS | | | | |
| $O_2^{\bullet-}$ | NA | NA | 11.5 ± 0.6 | 14.2 ± 0.4 |
| $H_2O_2$ | 29.4 ± 0.2%[b] | 894 ± 3 | 426 ± 7 | 509 ± 6 |
| HOCl | 47.1 ± 0.6 | 25.3 ± 0.4 | 0.39 ± 0.01 | 0.10 ± 0.01 |
| RNS | | | | |
| $^{\bullet}NO$ | 36.1 ± 0.3%[b] | 485 ± 2 | 18 ± 1 | 0.15 ± 0.01 |
| $ONOO^-$ | 167 ± 5 | 53 ± 1 | 2.64 ± 0.06 | 0.122 ± 0.004 |
| $ONOO^-$ [c] | 35 ± 1 | 20.6 ± 0.3 | 4.9 ± 0.1 | 0.121 ± 0.005 |

[a] $IC_{50}$ = inhibitory concentration, *in vitro*, to decrease the oxidizing effect of each reactive species by 50% (mean ± standard error of the mean, SEM). NA = no activity was found up to the highest tested concentration (1000 μg $mL^{-1}$). [b] Scavenging effect (%) (mean ± standard error of the mean, SEM) at 1000 μg $mL^{-1}$. [c] Assay carried out in the presence of $NaHCO_3$ (25 mM) to simulate the physiological concentration of $CO_2$.

$mL^{-1}$) (Table 3). The seed extract also exhibited a higher scavenging efficiency against $H_2O_2$ than quercetin (509 μg $mL^{-1}$) and the peel extract of *P. cattleianum* fruit,[4] but lower than the hydrophilic extracts of murici (228 μg $mL^{-1}$)[5] and the *V. cauliflora* plant (medicinal plant from Amazonia) ($IC_{50}$ from 106 to 401 μg $mL^{-1}$).[31]

The majority of $H_2O_2$ produced by phagocytes (neutrophils and monocytes) is used by the myeloperoxidase (MPO) enzyme to catalyse the oxidation of $Cl^-$, yielding HOCl. HOCl has been considered as a strong pro-inflammatory agent, and consequently it has been implicated in several diseases associated with chronic inflammation, such as atherosclerosis, ischemia-reperfusion renal injury, multiple sclerosis, Alzheimer's disease and some cancers.[32,33] This reactive species presents a very fast reaction rate with various compounds in biological systems, such as sulfhydryl, polyunsaturated fatty acids, DNA pyridine nucleotides and amino acids, and its toxicity has been measured to be between 100 to 1000 times higher than that of $O_2^{\bullet-}$ and $H_2O_2$.[34] Our results suggest that all *C. bracteosa* extracts have high potential to scavenge HOCl (Table 3 and Fig. 3c) with the seed extract as the most efficient one ($IC_{50}$ = 0.39 μg $mL^{-1}$), followed by the shell and pulp extracts. All the extracts presented higher scavenging capacities against HOCl than the freeze-dried extracts of *Cytisus scoparius* (56 to 60 μg $mL^{-1}$)[35] and the ethanol or ethyl acetate/ethanol extracts of *C. villosum* pulp (199 and 299 μg $mL^{-1}$, respectively),[3] but lower activities than quercetin (0.10 μg $mL^{-1}$).

Not only are ROS involved in the oxidative stress, but RNS are also known to interfere with the biological activity of several molecules, which may affect the shelf-life and the quality of food,[36] as well as being implicated in several human diseases.[37] Therefore, research strategies directed to searching for isolated compounds or plant extracts that act as natural antioxidants against RNS have assumed an important role in the modern science. Regarding this issue, $^{\bullet}NO$ is produced by the nitric oxide synthase (NOS) enzyme, by the conversion of L-arginine to L-citrulline,[37] and at low concentrations $^{\bullet}NO$ exhibits important activity in physiological conditions. However, if the production exceeds normal levels, it can cause harmful effects in the tissues leading to serious inflammatory conditions, as well as being involved in endotoxic shock.[37] As can be seen in Table 3, among all *C. bracteosa* extracts, the seed extract was, by far, the most active against $^{\bullet}NO$ ($IC_{50}$ = 18 μg $mL^{-1}$) (Fig. 3d), with a higher scavenging capacity than seed extracts of sesame (*Sesamun indicum*) (98–238 μg $mL^{-1}$), α-tocopherol (57 μg $mL^{-1}$),[38] extracts of some fruits used in traditional Indian medicine (*Terminalia chebula*, *Terminalia belerica* and *Emblica officinalis*) (33–41 μg $mL^{-1}$) and curcumin (91 μg $mL^{-1}$).[39] However, the $^{\bullet}NO$ scavenging capacity of *C. bracteosa* seed extracts was lower than that found for quercetin (0.15 μg $mL^{-1}$), and also less efficient than *V. cauliflora* extracts (0.9 to 3.6 μg $mL^{-1}$),[31] extracts of *P. cattleianum* fruit (2–7 μg $mL^{-1}$)[4] and infusion, decoction and hydroalcoholic extracts of artichoke leaves (5.5–11 μg $mL^{-1}$).[29]

The toxicity of $^{\bullet}NO$ is related to its high concentration in biological systems and in the presence of $O_2^{\bullet-}$ a highly oxidising species is formed: $ONOO^-$. This RNS has been shown to oxidize a variety of biomolecules including thiols, lipids, proteins, carbohydrates, DNA and has been implicated in the development of some diseases, including arteriosclerosis, cardiovascular diseases, inflammation, ischemia-reperfusion, cancer, diabetes and neurodegenerative disorders, such as Alzheimer's or Parkinson's diseases.[37] The $ONOO^-$-scavenging capacity of the *C. bracteosa* seed extract, in the absence (2.64 μg $mL^{-1}$) or presence of $NaHCO_3$ (4.9 μg $mL^{-1}$), was superior to that found for the shell and pulp extracts (Fig. 3e and f). The evaluation of the scavenging capacity of $ONOO^-$ in the presence of $NaHCO_3$ is important because, under physiological conditions, the reaction between $ONOO^-$ and $CO_2$ is predominant[40] and may lead to the formation of further reactive species that are also responsible for the nitration and oxidation reactions observed *in vivo*. These reactive species have

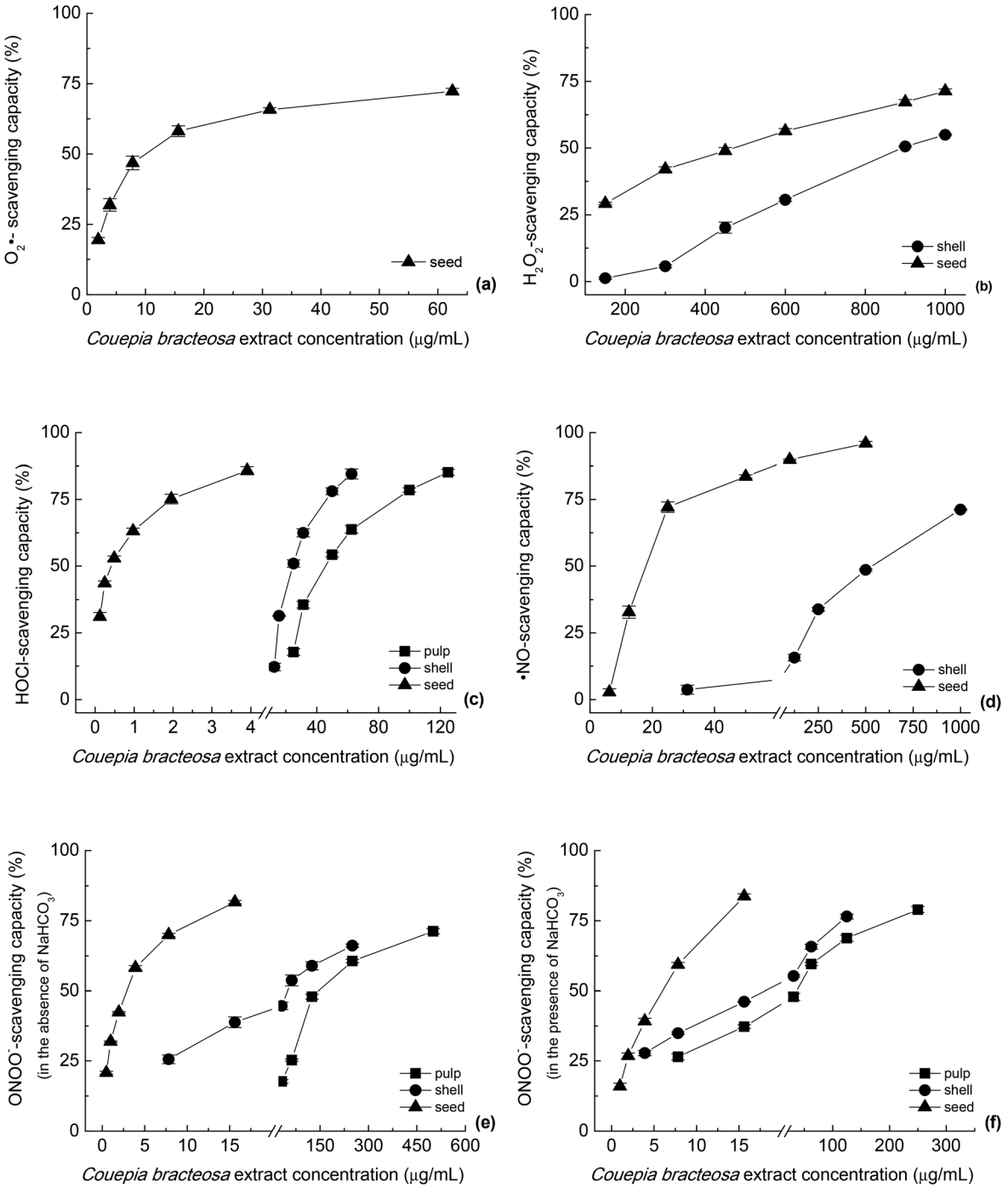


**Fig. 3** Scavenging capacities of the pulp, shell and seed extracts of *Couepia bracteosa* fruits against (a) superoxide radical ($O_2^{\bullet-}$), (b) hydrogen peroxide ($H_2O_2$), (c) hypochlorous acid (HOCl), (d) nitric oxide ($^{\bullet}NO$), and (e) peroxynitrite ($ONOO^-$) in the absence and (f) presence of $NaHCO_3$. Each point shows the standard error of the mean (SEM) bars and represents the values from four experiments.

the ability to oxidize a variety of biomolecules (thiols, lipids, proteins, carbohydrates, DNA, among others) *via* complex mechanisms of oxidation reactions which are strongly pH dependent.[37] An interesting effect was observed in the $ONOO^-$-scavenging effect of both the shell and pulp extracts of *C. bracteosa*, where they were more efficient in scavenging $ONOO^-$ in the presence of $NaHCO_3$ (20.6 and 35 μg $mL^{-1}$, respectively) than in its absence (53 μg $mL^{-1}$ and 167 μg $mL^{-1}$, respectively). The efficiency of seed extracts, in the absence and presence of $NaHCO_3$, was higher than the extracts of *P. cattleianum* fruit,[4] *V. cauliflora* fruit,[31] artichoke laves[29] and a hydrophilic extract of *B. crassifolia*.[5] In contrast, quercetin

 

(Table 3) showed a higher $ONOO^-$-scavenging capacity than all *C. bracteosa* extracts, in the absence or presence of $NaHCO_3$. Therefore, as the *C. bracteosa* extracts could scavenge $ONOO^-$, both in the absence and presence of $NaHCO_3$, they are also supposed to exhibit scavenging capacity against other reactive species, such as $^{\bullet}NO_2$ and $CO_3^{\bullet-}$.

## 4. Conclusion

For the first time, the profiles of phenolic compounds and carotenoids of extracts obtained from *C. bracteosa* fruits were reported, as well as their antioxidant capacities against the oxidizing effects of ROS and RNS of physiological importance. The seed extract was the most efficient one against all ROS and RNS probably due to the presence of apigenin sulphate, which was not detected in the other extracts. It is worth noting that, although all extracts have presented scavenging capacity against the tested ROS and RNS, in a concentration-dependent manner, they presented the highest efficiency against $^{\bullet}NO$ and $ONOO^-$ with $IC_{50}$ values in the low μg $mL^{-1}$ range. Thus, the extracts of *C. bracteosa* fruits may be considered as a promising source of bioactive compounds with high antioxidant properties exhibiting great potential for application in the pharmaceutical, cosmetic and food industries.

## Conflict of interest

The authors have declared no conflicts of interest.

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